Validation & Assay Performance Summary

CellSensor® AP1-*bla* A375 Cell Line

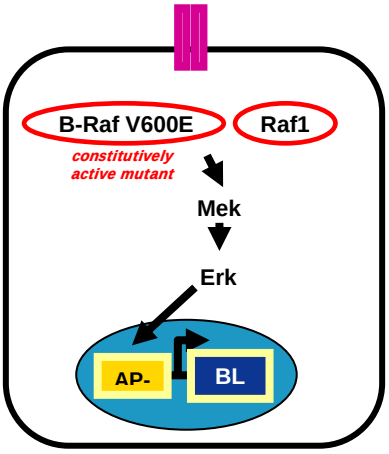
Cat. no. K1660

CellSensor® Cell-Based Assay Validation Packet

This cell-based assay has been thoroughly tested and validated by Invitrogen and is suitable for immediate use in a screening application. The following information illustrates the high level of assay testing completed and the validation of assay performance under optimized conditions.

Pathway Description

The RAF gene family (RAF1, A-RAF and B-RAF) encodes closely related serine/threonine protein kinases that are important effectors of Ras activation. Raf1 and A-Raf are rarely mutated, whereas mutations in B-Raf gene are common in human cancer, especially in melanoma. B-Raf is mutated in about 70% of human melanomas, 35-70% of papillary thyroid carcinomas, and less commonly in lung and colorectal carcinomas. Mutations are mostly in the B-Raf kinase domain and, in melanomas, the vast majority are V600E missense mutations leading to activation of B-Raf kinase. The constitutive active B-Raf V600E can directly lead to the activation of Mek/MapK signaling pathway. Therefore, inhibition of B-Raf/Mek/MAPK signaling could be a potential way for treating melanomas and other tumors with B-Raf mutation.



Cell Line Description

The CellSensor® AP1-*bla* A375 cell line contains a beta-lactamase reporter gene under control of the AP1 response element stably integrated into A375 cells. A375 cells are human melanoma cancer cells that contain endogenous B-Raf mutation V600E resulting in constitutively active B-Raf kinase activity. The CellSensor® AP1-*bla* A375 cell line is a clonal population isolated based on constitutive expression of beta-lactamase by flow cytometry. This cell line has been validated with various Raf small molecular inhibitors as well as B-Raf Stealth™ RNAi. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, compound incubation time, and substrate loading time and validated for Z' and IC_{50} concentrations of Raf1 Inhibitor I.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Raf1 Inhibitor I dose response under optimized conditions (n=3)**

   Raf1 Inhibitor I IC_{50} = 0.62 µM
   Z'-Factor (untreated cells) = 0.73
   Response Ratio = 5.5

   Recommended cell no. cells/well = 10,000
   Recommended [DMSO] = 0.0-1.0%
   Recommended Compound incubation time = 17 hrs

2. **Alternate Stimuli**
   N/A

3. **Small molecule inhibitor Testing**
   See Compound Panel

4. **Knockdown by Stealth™ RNAi**

5. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

6. **Assay performance with variable cell number**

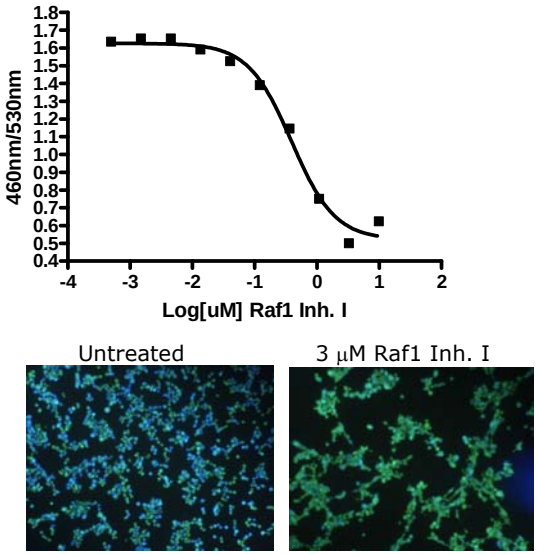
7. **Assay performance with variable stimulation time**

8. **Assay performance with variable substrate loading time**

9. **Assay performance with variable DMSO concentration**

Raf1 Inhibitor I Dose Response

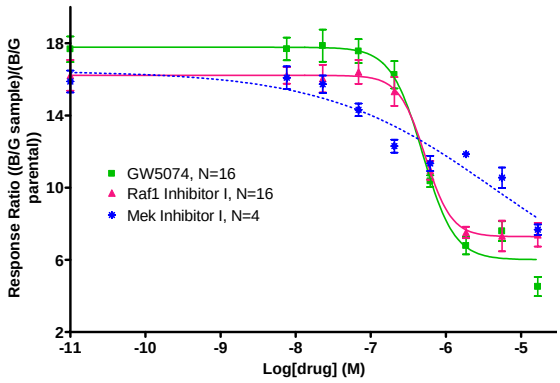
Figure 1 — Raf1 Inhibitor I dose response under optimized conditions



AP1-*bla* A375 cells (10,000 cells/well) were plated in a 384-well format and were treated with the indicated concentrations of Raf1 Inhibitor I (EMD #553008) in the presence of 0.5% DMSO for 17 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460nm/530nm ratio plotted for the indicated concentrations of Raf1 Inhibitor I (n=16 for each data point). Images of untreated and 3 µM Raf1 Inhibitor I treated cells are shown in the bottom.

Compound Panel

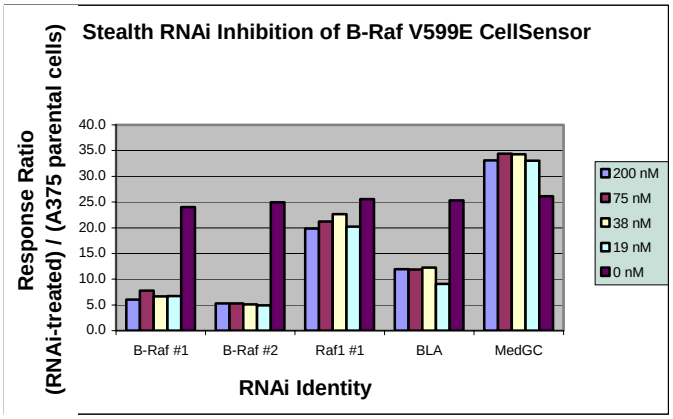
Figure 2 — Dose Responses of various small molecular inhibitors



AP1-*bla* A375 cells (10,000 cells/well) were plated in a 384-well format and treated with the indicated concentrations of Raf1 Inhibitor I (EMD #553008), GW5074 (Biomol #EI307), and Mek Inhibitor I (EMD #444937) for 17 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratio ((460nm/530nm ratio of drug treated cells)/(460nm/530nm ratio of A375 parental cells)) plotted for the indicated concentrations of the inhibitors (N for each data point is shown in the graph legend; means and standard deviations are plotted for each point).

Knockdown by B-Raf Stealth™ RNAi

Figure 3 — B-Raf Stealth™ RNAi inhibited beta-lactamase expression of AP1-*bla* A375 cells



The AP1-bla A375 cells were plated at 5,000 cells per well in 96-well tissue culture plates and incubated overnight in OptiMEM assay media, and then transfected for 24 hours with the indicated concentrations of Stealth RNAi (Invitrogen product numbers are B-Raf #1, 12936-02(RNAi Duplex1; B-Raf #2, 12936-02(RNAi Duplex2); Raf1 #1, 12935-033(RNAi Duplex1); BLA; MedGC, 12935-300) using the RNAi protocol for Lipofectamine2000 as recommended. The media was then replaced with fresh OptiMEM assay media, and the cells incubated for another 48 hours. Cells were then loaded with BLA substrate (plus solution D) for 3 hours and the blue/green (B/G) ratio measured on a Safire 2 plate reader. Results are expressed as the B/G ratio of the RNAi-treated (or mock transfected) cells divided by the B/G ratio of parental A375 cells. Med GC RNAi is a scrambled sequence oligo used as a negative control for non-specific RNAi effects, and it is not expected to knock down B-Raf signaling. Treatment with BLA RNAi should decrease the AP1-bla signals detected. B-Raf, Mek1 and Raf1 are all components of the MAPK pathway that feeds into the AP1 response element. Two different Stealth™ oligonucleotides targeted against B-Raf severely disrupt the constitutive AP1 activity in these cells demonstrating the strong dependence on the constitutive activity of the V599E B-Raf allele for the observed constitutive beta-lactamase expression.

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 20 and 80% confluence.

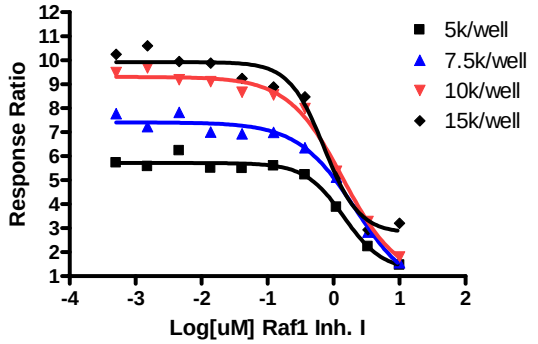
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM w/ GlutaMAX™	90%	--	—
OptiMEM	--	99.5%	
Dialyzed FBS	10%	0.5%	—
HEPES	--	10 mM	
NEAA	--	0.1 mM	—
Sodium Pyruvate	--	1 mM	
Penicillin (antibiotic)	100 U/ml	100 U/ml	—
Streptomycin (antibiotic)	100 µg/ml	100 µg/ml	—
Blasticidin (antibiotic)	5 µg/ml	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

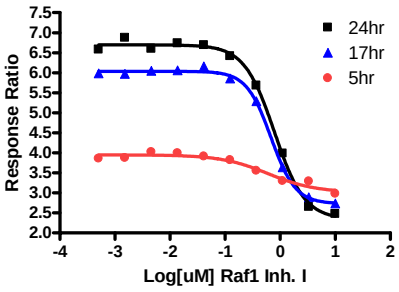
Figure4 — Raf1 Inhibitor I dose response with different plating cell numbers/well



AP1-*bla* A375 cells were plated with indicated number of cells/well in a 384-well format in assay medium. They were treated with indicated concentration of Raf1 Inhibitor I (EMD Cat.No. 553008) for 17 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 hours. Fluorescence emission values at 460 nm and 530 nm for the various cell numbers were obtained using a standard fluorescence plate reader and the Response Ratios were calculated as the ratio of blue/green ratio of the each sample vs blue/green ratio of 100 μM Clavulanate treated cells and plotted for each cell number. (n=8 for each data point). (Note: clavulanate directly inhibits beta-lactamase activity and is used here to set base line control.)

Assay Performance with Various Compound Incubation Time

Figure 5 – Raf1 Inhibitor I dose responses with various incubation times

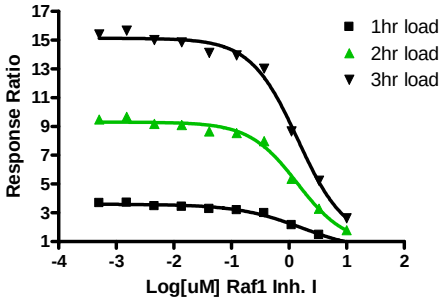


	24hr	17hr	5hr
BOTTOM	2.342	2.732	3.012
TOP	6.695	6.037	3.950
LOGEC50	-0.1026	-0.1659	-0.2170
HILLSLOPE	-1.587	-1.956	-1.011
EC50	0.7896	0.6825	0.6068

AP1-*bla* A375 cells were plated with indicated amount of Raf1 Inhibitor I in a 384-well format in assay medium for 5, 17 and 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios were calculated as the ratio of blue/green ratio of the each sample vs blue/green ratio of 100 μM Clavulanate treated cells. (n=8 for each data point).

Assay Performance with Variable Substrate Loading Time

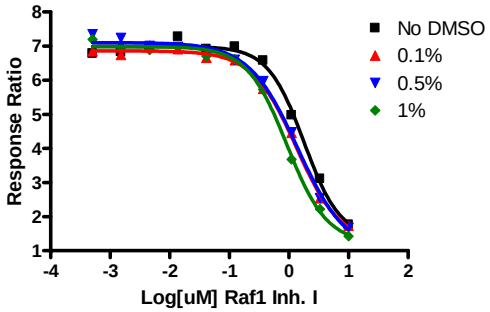
Figure 6 — Raf1 Inhibitor I dose response with various substrate loading times



AP1-*bla* A375 cells were incubated with indicated amount of Raf1 Inhibitor I in a 384-well format in assay medium for 17 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 1, 2 and 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios were calculated as the ratio of blue/green ratio of the each sample vs blue/green ratio of 100 μM Clavulanate treated cells. (n=8 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 7 – Raf1 Inhibitor I dose responses with 0, 0.1, 0.5 and 1% DMSO



AP1-*bla* A375 cells were incubated with indicated amount of Raf1 Inhibitor I in a 384-well format in assay medium for 17 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 hours. Fluorescence emission values at 460 nm and 530 nm for each sample were obtained using a standard fluorescence plate reader and the Response Ratios were calculated as the ratio of blue/green ratio of the each sample vs blue/green ratio of 100 μM Clavulanate treated cells. (n=8 for each data point).